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ALLOZYME-BASED GENETIC VARIABILITY OF THE *DAPHNIA ATKINSONI–BOLIVARI* SPECIES COMPLEX (CLADOCERA: DAPHNIIDAE) IN THE HUNGARIAN GREAT PLAIN

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Allozyme polymorphism investigation was performed with the aim to evaluate some population genetic characteristics of two species, *Daphnia atkinsoni* Baird, 1859 and *Daphnia bolivari* Richard, 1888 (Cladocera: Daphniidae), of temporary aquatic habitats in the Hungarian Great Plain. The analysis showed that *D. bolivari* is not a distinct taxon, but is nested within *D. atkinsoni*. At the same time, remarkable within-species differentiation was revealed in *D. atkinsoni*; two presumable cryptic lineages were detected by our enzyme polymorphism investigation. The separation of the lineages is mainly due to the allozyme pattern at the AAT locus. Multivariate analysis of abiotic variables of the sampling sites revealed the relation between the genetic and ecological data, pH being the relevant variable explaining 81% of the genetic variability.

Key words: *Daphnia atkinsoni*, *Daphnia bolivari*, genetic diversity, allozyme, cryptic lineages.

INTRODUCTION

Much attention is paid to temporary waters nowadays since they provide opportunities to study the effect of local biotic and abiotic factors (Frisch *et al*. 2006, Waterkeyn *et al*. 2008), dispersal, connectivity and metapopulation dynamics (Bohonak & Jenkins 2003, Green *et al*. 2008) and climate change (Van Doorslaer *et al*. 2010) on zooplankton species and communities. At the same time temporary waters are threatened habitats since their existence is highly dependent on the local precipitation conditions and the hydrological regime, that has been altered drastically during the last centuries through river regulations and agricultural expansion worldwide.

The morphologically variable *Daphnia (Ctenodaphnia*) *atkinsoni* Baird, 1859 (Cladocera: Anomopoda: Daphniidae) is generally considered to be a warm-water species occurring in temporary aquatic habitats in Southern Europe and North Africa (Hudec 1981). Its occurrence in Northern Europe is sporadic, however a low temperature race of the species was reported from the UK (Johnson 1952) and the occurrence of *Daphnia atkinsoni* was recently recorded in Belgium also (Louette & De Meester 2004) where it rapidly colonised a

newly dug pool. The distribution and occurrence of *D. atkinsoni* in Hungary was summarised by Forró (1994). It was found in the plain to the east of the Danube in various small waterbodies such as rain pools, wheel tracks and in sodic waters, too. The species occurred mostly between February and June, the highest number of records is in April–May and in very few cases between September–November. There are no previous records of *D. bolivari* from Hungary because following other authors e.g. Flössner (1972) it was considered a synonym of *D. atkinsoni*. One of us (L.F.) following Alonso (1996) recorded *D. bolivari* in the Great Plain, however the finding remained unpublished.

Daphnia atkinsoni was described based on specimens hatched from dry mud collected in Israel. In July 1858 Edward Atkinson sent dried mud from the pool of Gihon in Jerusalem to Mr Denny, and this latter gentleman forwarded part of the mud to Baird, who received it in June 1859 (Baird 1859). He put the mud in water and by the middle of July he had five new species (one conchostracan, one cladoceran, two ostracods and one calanoid copepod). The cladoceran was *Daphnia atkinsoni*, its description was based on females only. About thirty years later Richard (1888) published the description of *D.* (*Ctenodaphnia*) *bolivari* Richard 1888 from Spain, later in his revision of the Cladocera he considered it as a variety of *D. atkinsoni* (Richard 1896). Type material of this species is present in the National Museum of Natural History, U.S.A. (Kotov & Ferrari 2010). Thereafter the two species were considered conspecific by Wagler (1925, 1935) and Gauthier (1927). This status has been accepted for a long time by various authors (*e.g*. Sramek-Husek 1964, Flössner 1972, Negrea 1984). Later Alonso (1980) regarded the *bolivari* form as a subspecies, *D. atkinsoni bolivari*. More recently Alonso (1991, 1996) adopted a different view and considered them as two distinct species. This was then quite widely accepted (e.g. Flössner 2000, Benzie 2005, Marrone *et al.* 2007). This distinction is largely based on the difference in the spination of the dorsal ridge and the head shield ("crown of thorns") and the size of the lateral lobe of the dorsal carina on the head. *Daphnia atkinsoni* was considered a highly variable species (Petkovski 1970, Hrbacek 1987), according to Benzie (2005) it is likely that *D. atkinsoni* and *D. bolivari* constitute morphological extremes of one taxon. Recently it has been shown based on sequences of the 12S ribosomal DNA and the cytochrome oxidase subunit I. (Petrusek *et al*. 2009) that the morphologically distinct *Daphnia bolivari* (Richard, 1888) is identical with *D. atkinsoni*.

The genetics of certain species of Cladocera, like for example *Daphnia magna* Straus, 1820, *Daphnia pulex* Leydig, 1860 and the *Daphnia longispina* group is extensively studied (Colbourne *et al.* 1998, De Gelas & De Meester 2005, Galimov *et al.* 2011, Yin *et al*. 2012) in temporary and permanent aquatic habitats, but genetic research on other species is scarce (Kotov *et al.* 2006,

Juračka *et al.* 2010). Therefore the number of species that can be the subject of molecular ecological studies is limited. Population genetic data based on allozyme studies on a single *D. atkinsoni* population were first published in 2007 (Louette *et al*. 2007), later microsatellite markers have been developed and the genetic structure of one Belgian and one Spanish population was reported for *D. atkinsoni* (Ortells *et al.* 2009). With the investigation of mitochondrial genes it was concluded that *D. atkinsoni* and *D. bolivari* are not separate species (Petrusek *et al.* 2009). Our aim was to investigate the level of divergence between *D. atkinsoni* and *D. bolivari* based on nuclear markers (allozymes). We also aimed to give population genetic measures based on allozyme markers for the species complex, since to our knowledge data were published only for one population of *D. atkinsoni* from Belgium so far (Louette *et al*. 2007).

As the general population genetic theory predicts, the genetic diversity within a species in a large habitat is potentially larger than within the same species in a smaller habitat patch (Hartl & Clark 1989). The reason for this is that the larger habitats tend to be ecologically more diverse providing suitable habitats for more genotypes. The other reason is that larger habitats are able to maintain bigger populations that are potentially more diverse. The connection between habitat size and genetic diversity was detected by Michels *et al.* (2003), who observed a positive correlation between local genetic diversity and habitat size for *Daphnia obtusa* Kurz, 1874 and *Daphnia pulex* populations in Belgium, however, for *Daphnia curvirostris* Eylmann, 1887 the correlation was negative. We aimed to study the correlation between habitat size and genetic diversity in the case of *Daphnia atkinsoni*.

Factors affecting the build-up of microcrustacean communities were considered to have potential effect on the genetic structure of the *Daphnia* populations. Such factors were salinity (Waterkeyn *et al.* 2008), pH (Holt *et al*. 2003) and depth (Medley & Havel 2007). We aimed to study the genetic structure of the *D. atkinsoni* populations in connection to these relevant abiotic characteristics of the randomly choosen sampling sites.

MATERIALS AND METHODS

We collected zooplankton samples by net tows (85 μm mesh size) from seven temporary waterbodies in the Hungarian Great Plain (Fig. 1 and Table 1). Among our sampling sites there is one bomb crater (Ap1), originating from the 1950's, when the area was used as a rifle range. One sampling site, an inundated area, lied in an uncultivated field (KM3). Other sites were found on agricultural lands under cultivation, KM13 and P12 are flooded areas, Ko9 and S12 are rainwater pools, while Ko17 is a wheeltrack. In the year of our sampling (2006) there was an extreme amount of precipitation and a big number of pools filled up in the Great Plain. The age of our sampling sites is not known.

Table 1. Summarized data on the sampled populations. The name of the nearest town to the sampling site is given in the first row. Geographical coordinates (WGS84) of the sampling locations, sampling date (in 2006), species assignments (D. atk. – *Daphnia atkinsoni*, D. bol. – *Daphnia bolivari*) and the number of individuals studied (N) is given in the following rows. Abiotic parameters: Cond – conductivity (µS/cm), Sal – salinity (g/l), depth (cm), surface area (m^2).

	KM13	KM3	Ko9	Ko17	Ap1	S12	P12
Town	Kardoskút		Konyár		Apaj	Izsák	Besenyőtelek
N	46°28′18″	46°28′16″	47°17′58″	47°19′49″	47°07′24″	46°46′11″	47°41′33″
E	20°35′33″	20°35′27″	21°44′08″	21°44′03″	19°07′50″	19°24′04″	20°26′41″
Date	04. April	04. April	11. May	11. May	30. March	25. April	20. April
Species	D. atk.	D. atk.	D. bol.	D. bol.	D. atk.	D. atk.	D. atk.
N	33	22	22	33	44	44	44
Cond	348	738	650	1667	3610	739	1445
Sal	0	0.1	0.1	0.7	1.8	0.1	0.5
pH	8.09	7.59	8.15	8.64	8.71	8.79	8.84
depth	50	60	15	10	70	20	30
surface	2000	3000	375	4	41	80	7500

Zooplankton was placed into a white plastic tray on the spot, and individuals were collected into cryotubes using a pipette. Samples were frozen in liquid nitrogen on the sampling site, carried to the laboratory and stored at -70°C until further processing by cellulose acetate gelelectrophoresis. We aimed to collect 40 animals per population whenever animals were abundant enough to reach this number. Beside the collection of zooplankton samples we measured pH, conductivity and salinity by a WTW Multiline P3 device. Water depth was measured with the help of a measuring tape. Surface area was estimated based on direct measurements by tape measure for pools not exceeding 40 m in length, while surface area of pools over this size was estimated by eye.

Individuals were identified under a microscope before proceeding to the gelelectrophoresis. Based on the identification two populations of *D. bolivari* and 5 populations of *D. atkinsoni* have been included in the analysis (Table 1). We used the Helena Super Z-12 Applicator kit (Helena Laboratories, Beaumont, Texas) and the ZipZone Chamber (Helena Laboratories) for the cellulose acetate gelelectrophoresis, that was carried out as described in (Hebert & Beaton 1993). Individuals of a parthenogenetically reared *Daphnia magna* clone were used as markers in each run in the ninth position on the gel. We tested the variability of the PGI, PGM, MDH, AAT, MPI, AO, LDH and ADH loci on at least eleven individuals and finally

carried out invetigations only on the PGI, PGM, AAT and MDH loci. The number of individuals per population can be found in Table 1.

Population genetic analyses of the collected data were conducted in TFPGA (Miller 1997). We calculated Wright's F- statistics based on the method of Weir and Cockerham (1984) with 95% confidence intervals by 1000 iterations for the total dataset. Wright's F – statistics were calculated with the same parameters also for two subsets of the data, corresponding to two putative cryptic lineages, that resulted from the UPGMA analysis (group 1: KM13, Ko17, Ko9, KM3 and group 2: P12, Ap1, S12). The UPGMA analysis is based on Nei's original distances. A 3D factorial correspondence analysis (FCA) was performed in GENETIX (Belkhir *et al*. 1996–2004) for the visualization of the genetic variation across populations. The number of the multilocus genotypes (MLG) and clonal diversity expressed as the Simpson index was calculated in Hwclon (J. Vanoverbeke, unpubl. data).

We calculated Pearson's product-moment correlation between the habitat size and the genetic diversity for the populations in R (R Development Core Team 2010). This analysis was done for the total dataset and for two subsets of the populations, as in the case of Wright's F-statistics.

We generated an ecological distance matrix in PC ORD (McCune & Mefford 1999) using the Euclidean distances calculated based on the environmental variables (Table 1). A Mantel test was performed on the genetic distance matrix and the ecological distance matrix with 9999 permutations in PC ORD.

To examine the relevant abiotic parameters we performed a multivariate analysis on the basis of Nei's original genetic distances with forward selection of explanatory variables in a linear regression model using 4999 permutations with the DISTLM forward 1.3 (Anderson 2003) program. Environmental data have been $\log_{10}(x+1)$ transformed prior to the

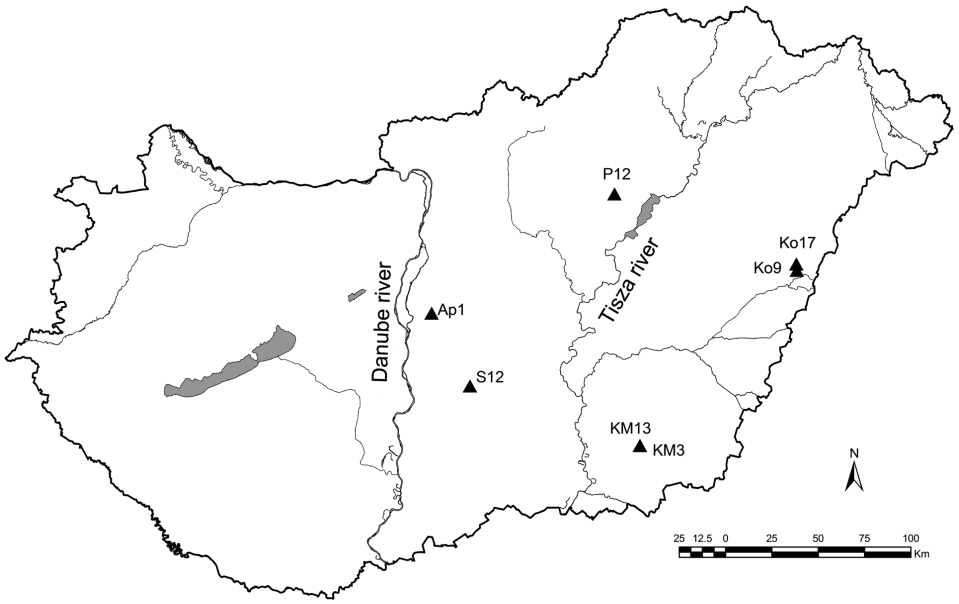

Fig. 1. Sampling sites on the map of Hungary.

Table 2. Allele frequencies and heterozygosity (H, direct count) per alleles for the seven studied populations. H_{av} – average heterozygosities based on direct counts, H_{ex} – expected average heterozygosities, MLG – number of the multilocus genotypes, CD – clonal diversity in the population.

	KM13	KM3	Ko9	Ko17	Ap1	S12	P12
PGI 1	1	1	1	1	1	0.75	1
PGI 2	0	0	0	0	0	0.25	0
H_{PGI}	0	0	0	0	0	0.27	0
PGM 1	1	1	1	1	0.89	0.89	1
PGM 2	0	0	0	0	0.11	0.11	0
H_{PGM}	0	0	0	0	0.14	0.12	0
AAT 1	0.79	1	1	0.82	0	0	0
AAT 2	0.21	0	0	0.18	0.87	0.55	0.67
AAT 3	0	0	0	0	0.13	0.45	0.33
H_{AAT}	0.42	0	0	0.3	0.2	0.75	0.31
MDH 1	0.94	0.8	1	1	1	1	1
MDH 2	0.06	0.2	0	0	0	0	0
H_{MDH}	0.13	0.41	0	0	0	0	0
H_{av}	0.137	0.102	0	0.076	0.085	0.286	0.079
H_{ex}	0.113	0.081	0	0.074	0.105	0.266	0.110
MLG	4	2	1	3	5	9	3
CD	1.664	1.936	1	2.104	2.397	6.259	2.547

analysis to approach normality. Salinity was omitted from this analysis since it displayed significant correlation with conductivity.

RESULTS

In the prior tests of eleven individuals the PGI, PGM, MDH and AAT loci showed variation and the MPI, AO, LDH and ADH loci were monomorphic, therefore these were omitted from further cellulose-acetate gelelectrophoresis. We observed two alleles at the PGI, PGM and MDH loci while three alleles occured at the AAT locus. Observed allele frequencies and heterozygosities for each locus for the seven studied populations along with the number of the multilocus genotypes per population and the clonal diversity are given in Table 2. The Ko9 population was monomorphic at each locus. In the case of the PGI locus the S12 population carried a private allele (PGI 2) at the frequency of 0.25.

Table 3. Wright's F-statistics over the total dataset and two subsets. The subsets correspond to populations of the two clusters of the UPGMA and FCA analyses (KM13, Ko17, Ko9, KM3 and P12, Ap1, S12).

	F_{IT}	F_{ST}	F_{IS}
Total dataset	0.49	0.47	0.04
Upper and	0.55	0.57	0.34
lower C.I.	0.16	0.1	-0.09
S.D.	0.16	0.28	0.15
KM13, Ko17, Ko9, KM3	-0.02	0.11	-0.15
Upper and	-0.01	0.12	-0.13
lower C.I.	-0.03	0.1	-0.17
S.D.	0.01	0.01	0.02
P12, Ap1, S12	0.23	0.13	0.11
Upper and	0.45	0.24	0.36
lower C.I.	0.08	0.05	-0.04
S.D.	0.18	0.04	0.2

The *D. bolivari* populations (Ko9 and Ko17) were clustered between two *D. atkinsoni* populations (KM13 and KM3) in the UPGMA clustering of Nei's original genetic distances (Fig. 2). The other cluster, formed by the P12, Ap1 and S12 populations, diverged at the level of 0.218 Nei's original distance from the first group (Fig. 2). This separation is mainly due to the AAT locus, where the AAT1 allele was found at high frequencies in the first cluster but was missing in the second cluster, whereas the AAT3 allele was found only in the second cluster but not in the first one (Table 2).

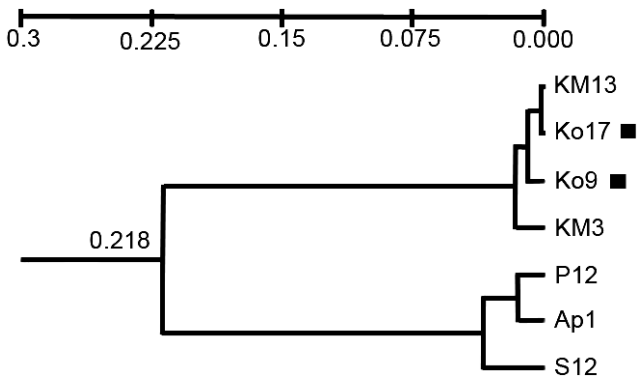

Fig. 2. UPGMA clustering of Nei's original genetic distances between populations of *Daphnia atkinsoni* and *D. bolivari*. The morphological *D. bolivari* populations are marked with black squares (Ko17 and Ko9).

Table 4. Results of the multivariate multiple regression analysis of pH, depth, conductivity and surface of the sampling sites, based on Nei's original genetic distances. '–' did not add to the explanation of cumulative variation.

abiotic variable	explained variation	p
pH	0.8048	0.023
depth	0.2250	0.984
conductivity	0.0015	0.470
surface	–	

Wright's F_{ST} indicated very great genetic differentiation among populations (Table 3) when calculated for the total dataset ($F_{ST, total} = 0.47$). Within the population subsets corresponding to the two clusters of the UPGMA analysis, genetic differentiation was moderate ($F_{ST, KM13, Ko17, Ko9, KM3} = 0.11$ and $F_{ST, P12, Ap1, S12} = 0.13$).

In the factorial correspondence analysis of the allozyme data across populations the first axis explained 71.99%, the second axis explained 16.65% and the third axis explained 6.34%, cumulatively 94.98% of the variation. Figure 3 depicts the FCA analysis, the same groups as in the UPGMA analysis (P12, Ap1, S12 and Ko9, Ko17, KM3, KM13) were separated along the first axis of the FCA.

The genetic and ecological distances were significantly correlated (Mantel test $r = 0.963$, $p < 0.001$), and the multivariate multiple regression analysis pointed out pH as the relevant ecological parameter explaining 80.5 % of the variation in the genetic distances (Table 4). The first cluster of the *D. atkinsoni*

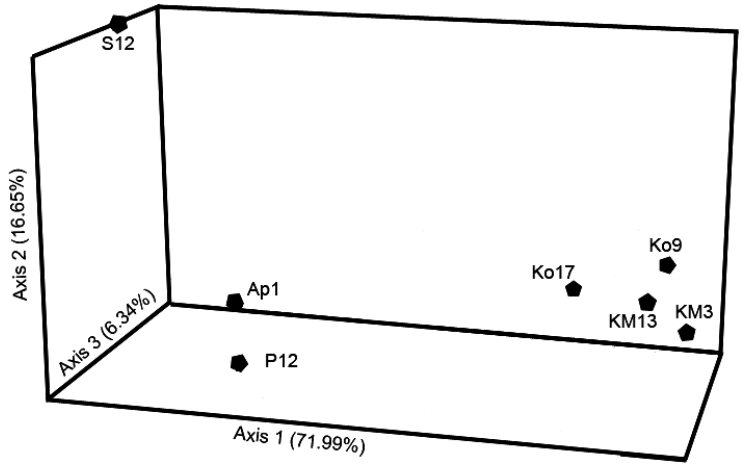

Fig. 3. FCA analysis of the allozyme data across populations of *Daphnia atkinsoni* and *D. bolivari*.

species complex in our study occurred at pH ranging from 8.71 to 8.84 while the second cluster preferred lower pH within the range of 7.59–8.64 (Table 1).

Pearson's correlation between the habitat size and the clonal diversity of the populations was r = – 0.143 (p = 0.759) when all the seven investigated populations were included in the analysis. For the group of the KM13, KM3, Ko9 and Ko17 populations we obtained r = 0.247 (p = 0.753) and for the other group (P12, S12, Ap1) the correlation coefficient was r = -0.466 (p = 0.691).

DISCUSSION

Petrusek *et al.* (2009) concluded based on the *12S* and *COI* mitochondrial genes that the spined morphs do not form a distinct species, but are nested within *D. atkinsoni*. Our investigations were based on nuclear markers and confirmed the same result, the spined morphs did not form a distinct group in the UPGMA and FCA analyses (Figs 2 and 3). The crown of thorns of the *D. bolivari* specimens may be the result of a phenotypically plastic inducible defense mechanism against predators (Laforsch *et al*. 2009, Petrusek *et al.* 2009).

In the study of the mitochondrial *COI* and *12S* genes altogether four lineages of the *Daphnia atkinsoni* complex were found, and two of these occurred in Hungary (Petrusek *et al.* 2009). The detected high F_{ST} value for the total dataset and the moderate values for the divided data in our study, furthermore the UPGMA clustering of Nei's genetic distances and the FCA analysis also support the existence of two *D. atkinsoni*- like lineages in Hungary. However the existence of more lineages within the country is also possible and investigation of the entire range of the species would be necessary to clarify how many cryptic lineages there are in the *Daphnia atkinsoni* species complex.

For a Belgian population of *D. atkinsoni* only the PGM locus was polymorphic (Louette *et al.* 2007), but in our study the PGI, PGM, AAT and MDH loci proved to be variable in the species, however not to a great extent. Both the Belgian and the Hungarian specimens presented two alleles at the PGM locus. Variability was not detected on other tested loci (MPI, AO, LDH and ADH).

The geographical distribution of the two clusters that were detected in the UPGMA and FCA analyses correspond to the west (Ap1, S12, P12 group) and to the east (KM3, KM13, Ko9, Ko17 group) from the Tisza river (Fig. 1), however the geographic barrier role of the river is unlikely in the case of the *Daphnia atkinsoni*-group, because *D. atkinsoni* is presumably a good passive long-range disperser, as it was observed in a study on zooplankton composition in a newly created pool in Belgium (Louette & De Meester 2004).

Geographically in the Duna–Tisza Interfluve the PGI2, PGM2 and AAT3 alleles occured, in the Tiszántúl region (that is to the East from the Tisza river) the MDH2 and AAT1 alleles were found and the PGI1, PGM1, AAT2 and

MDH1 alleles were ubiquitous to both regions. Investigation of further populations would be necessary to clarify the geographic pattern in the *Daphnia atkinsoni* complex.

Louette *et al.* (2007) studied a newly established *D. atkinsoni* population during three consecutive years in Belgium. In comparison to their results the observed clonal diversity in the Hungarian populations is lower or nearly equal, except for the S12 population. However, Louette *et al.* (2007) investigated more individuals per population and more loci than we did, both of which potentially increase the observed clonal diversity. In our survey, that was based on four enzyme loci, in three out of seven *D. atkinsoni* populations the number of observed MLGs (Table 2) was higher than in the Belgian *D. atkinsoni* population (Louette *et al.* 2007). Observed clonal diversity (6.259) and the observed number of multilocus genotypes (9) in the S12 population was strictly high in comparison to other Hungarian and the Belgian (Louette *et al.* 2007) *D. atkinsoni* populations, or even to a *D. obtusa* population, where the highest number of observed MLGs was seven and the clonal diversity associated to it was 4.19 (Louette *et al.* 2007) based on the variation at seven loci. Probably the genetically highly diverse S12 population existed for a long time, that allows for the immigration of new clones into the habitat. Genetic diversity of the Ko9 *D. atkinsoni* population was the minimum (1) with one observed multilocus genotype. This population might be newly established by a recent coloniser.

Michels *et al*. (2003) observed a positive correlation between local genetic diversity and habitat size for *Daphnia obtusa* and *Daphnia pulex* populations in Belgium. We could not detect a significant correlation between the habitat size and the genetic diversity of the *D. atkinsoni* populations, neither for the total dataset nor for the subsets corresponding to presumable cryptic lineages in *D. atkinsoni*. However, one possible reason to explain this is that the extreme precipitation conditions in 2006 resulted in larger pools than the usual size (for example KM13, KM3 and P12 were large, inundated areas) and this might mask the obtained pattern. Beside this, our sample size in the case of the divided dataset was too low to draw proper conclusions.

The genetic structure of the populations was heavily dependent on the pH (80.5% explained variation) and the genetic and ecological distance matrices were correlated. The effect of changes in pH conditions are frequently targeted in connection to freshwater acidification (Brett 1989, Schartau *et al*. 2001) and pH was identified as a determinant of zooplankton species richness and composition. Zooplankton communities become different around pH 5–6 compared to circumneutral pH (Holt *et al*. 2003). In our survey the pH conditions were slightly alkaline therefore the found pattern in genetic composition is difficult to explain.

Both our survey and the investigation of Petrusek *et al*. (2009) detected two cryptic lineages of *Daphnia atkinsoni* in Hungary, however, this number

might easily be more, since the sample size was not high in either case. A future study based on mitochondrial DNA investigation combined with the use of microsatellite markers (Ortells *et al.* 2009) could detect more cryptic lineages and genetically more diverse populations in the country.

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